THE NATURE OF THE INCLUSION BODY OF VERRUCA VULGARIS: A HISTOCHEMICAL STUDY OF NUCLEOTIDS*

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Verrucae are of interest to the clinician as a common disease of the skin and are important to the laboratory investigator as tumours induced by a filterable infectious agent. This paper reviews the established evidence for much of our present knowledge of warts, and reports the results of an investigation of some of the important chemical changes which occur in the infected epithelial cells. The findings not only aid in making an accurate microscopic diagnosis of the disease, but also provide evidence for a mechanism of virus multiplication.

VIRUS AS THE ETIOLOGIC AGENT OF VERRUCAE

The contagious nature of warts was demonstrated by Jadassohn (6) who transmitted the disease by embedding wart particles into the skin of human subjects. In 31 of 86 attempts, warts appeared at the site of inoculation after an incubation period of 6 weeks to 5 months. Lanza (8) substantiated the infectiousness of warts by this method of implantation. Payne (10) reported the development of a sub-ungual wart one week following the use of the finger nail during removal of a patient's wart. Although these experiments were suggestive of the presence of an infectious agent, they did not rule out the possibility of the direct transfer of viable tumour cells.

In 1907 Ciuffo (3) produced warts on his own hand by the inoculation of a sterile, cell-free filtrate of wart material. Papillomatous growths, clinically diagnosed as warts, appeared after an incubation period of 5 months. Waelsch (16) in 1918 demonstrated experimentally that verruca vulgaris and condyloma acuminatum were caused by the same filterable agent; inoculation of an extract of condyloma into human skin produced a common wart, and when inoculated into mucous membrane produced condylomata. Serra (13) reported that inoculation of a sterile, cell-free filtrate of condyloma into human skin produced warts. He also felt, therefore, that the virus of condyloma acuminatum was identical with that of verruca vulgaris. In 1919 Wile and Kingery (17) repeated experiments similar to those of Ciuffo. They inoculated the skin of humans with a bacteriologically sterile, cell-free filtrate of wart material. Lesions appeared at the site of inoculation 4–5 weeks following infection. A biopsy of a lesion of 8 weeks' duration revealed the characteristic histologic features of verruca vulgaris. In a second series of experiments (18) they used a cell-free filtrate of first-generation experimentally induced warts, and were able to produce second-generation verrucae in six months. Ullman (15) postulated that the same etiologic agent causes condyloma acuminatum, verruca plana, and laryngeal papilloma. In his experiments, inoculation of a suspension of human laryngeal papilloma produced flat warts in scarified human skin and condylomata of the vaginal mucous membrane of dogs. A sterile cell-free suspension of the first and second generation human warts produced a third generation of flat warts in human skin in one to four months. To our knowledge, the transmission of laryngeal papilloma has not been confirmed, moreover, there is great doubt of the validity of the transmission of human warts to dogs. Not only have others failed to repeat his findings in dogs under proper

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conditions, but also he failed to rule out the possibility of the development of native dog verrucae which commonly appear following the traumatic manipulations of the type he used. In addition, it has not been possible to transmit dog or cattle warts to humans.

SPECIFIC CYTOLOGY OF VERRUCA

In many virus diseases of the skin specific cytologic and histologic changes are observed. The most significant feature is the appearance of a distinctive mass of inclusion material (inclusion body). It may be found in the cytoplasm of cells, such as those infected with vaccinia, variola, and molluscum contagiosum or in the nucleus as in varicella, herpes simplex and herpes zoster. In the literature of verruca, however, there has been little agreement as to the location of the inclusion material, and as a result, some authors have doubted its presence. An early investigator, Sangiorgi in 1915 (12), described multiple acidophilic intracytoplasmic inclusions occurring in the stratum granulosum and upper layers of the strata spinosum. In 1924 Lipschütz (9) gave a classical discourse on the architecture of verrucae vulgaris and described in great detail a basophilic intranuclear inclusion which was present in some young warts. These inclusions occurred in the strata spinosum and stratum granulosum. Some of the modern texts of dermatologic histopathology fail to describe adequately or even to mention the inclusion body of warts.

More recently, Hyden (5) conducted a microspectrographic and cyto-chemical investigation of virus-infected tissues. Ultraviolet photomicrographs of normal human epidermis and of skin infected with verruca vulgaris as well as other diseases, were taken at absorption maxima (2600A) of the nucleic acids. With this technic, the distribution of nucleic acids can be readily demonstrated within individual cells. In verruca vulgaris a great increase of nucleotides occurred in the nucleus. The changes observed consisted of a network which first appeared about the nucleoli, and later increased and ultimately filled the entire nucleus by fusion of the aggregates. This mass of newly formed, distinctive material corresponds to the cell inclusions described by Lipschütz. The following study was undertaken, therefore, to clarify the existence of the inclusion material of Lipschütz and to extend Hyden's investigation of its chemical constituents.

MATERIALS AND METHODS

Approximately 150 verrucae from 65 patients were obtained. They comprised 51 patients with verruca vulgaris, 2 with v. plana, 4 with v. filiformis, 2 with v. palmaris or plantaris, and 6 with condylomata acuminata. Specimens were obtained from both adults and children, the youngest being three years. In some cases previous therapy had been given, while in others, the verrucae were untreated. The duration of individual lesions studied ranged from three weeks to several years.

The excised warts were divided, when possible, into several specimens. Individual pieces of tissue were fixed in 10% formalin, or acetic acid Zenker's solution, or absolute alcohol. Sections were prepared in the routine way and stained with hematoxylin and eosin and with pyronine-methyl green. Those considered

to be of special interest were also stained with toluidin blue to demonstrate both the ribo- and desoxyribonucleic acids (RNA and DNA), and with the Feulgen stain[1], to demonstrate only the desoxyribonucleic acids (DNA) (4). Although many sections were studied from each wart, no attempt was made to prepare complete serial sections of each lesion. The histologic sections were examined and compared with other virus diseases of the skin, as well as with any other disease of the skin which might conceivably exhibit similar cytologic

FIG. 1. Verruca vulgaris. H & E × 75. The process of distending the nuclei with basophilic inclusion material begins in the deeper prickle cell layers. The large dense fully formed "inclusion bodies" are surrounded by a clear zone of "ballooning degeneration."

changes, such as senile keratoses, seborrheic warts, Darier's disease, Bowen's disease, callosities, and cornu cutaneum.

RESULTS

In all of the tissues examined, the only changes in individual cells which were distinctive for verrucae occurred in the nucleus. It was possible to demonstrate the unique intranuclear cytologic changes described by Lipschütz (9) and Hyden (5), but the cytoplasmic bodies described by Sangiorgi (12), Strauss (14), and others were seen also in non-verrucous tissues and were thought to be non-specific

[1] Courtesy of Dr. Herbert Mescon, Dept. of Dermatology, Univ. of Pennsylvania.

changes (Figs. 5, 6). In addition, the histochemical evidence suggests that the most significant alterations occur in the nucleus.

As Hyden demonstrated, the earliest abnormalities are seen in the deeper layers of the stratum spinosum (5). As the cells move to the surface of the skin changes become more marked (Figs. 1, 7, 9). The pronounced abnormalities, therefore, are usually found in cells of the upper stratum spinosum and stratum granulosum. These cells which represent the culmination of a dynamic process have nuclei which are completely filled with inclusion material (Figs. 2, 3, 4, 8, 10). Such a nucleus is two or three times the size of a normal epithelial cell

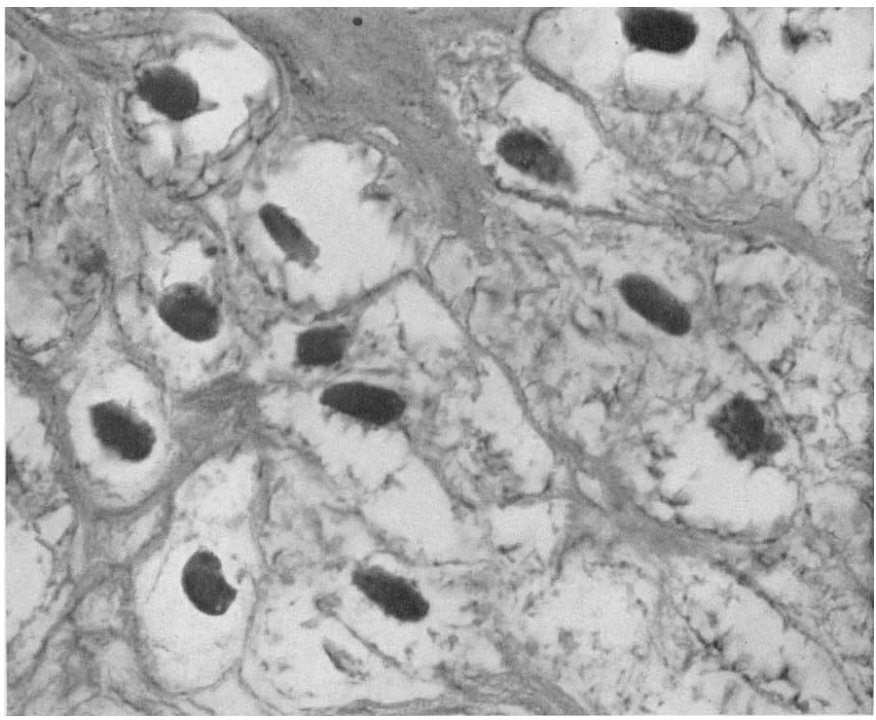

FIG. 2. Verruca vulgaris. H & E × 600. The cells containing "inclusion bodies" (densely filled nuclei) have a degenerated swollen cytoplasm.

nucleus. The normal fine structure of chromatin granules and nucleoli is completely replaced by a dense homogeneous mass. This inclusion material has the density of a pyknotic nucleus, but could be readily distinguished from a degenerate form by its full, rounded shape and its huge size in comparison with the small shrunken mass of a pyknotic nucleus.

The mature, enlarged, inclusion-filled nuclei can be seen readily under low power magnification. They appear as ovoid dense bodies and frequently are surrounded by a clear zone which is analogous to the "ballooning degeneration" described in other virus diseases of the skin (2) (Figs. 1, 2).

The warts removed from 65 patients were carefully studied for the presence

of the typical mature inclusion. Multiple sections were examined from each verruca. Table I is a summary of the findings and indicates that in only one half of the verrucae were inclusions found. In a number of instances multiple warts were examined from the same patient. One of these patients had inclusions in only 4 of 7 verrucae studied, another had inclusions in 2 out of 3 warts, and a third patient had them in only one out of 2 warts examined. These three patients had clinically typical verrucae vulgaris.

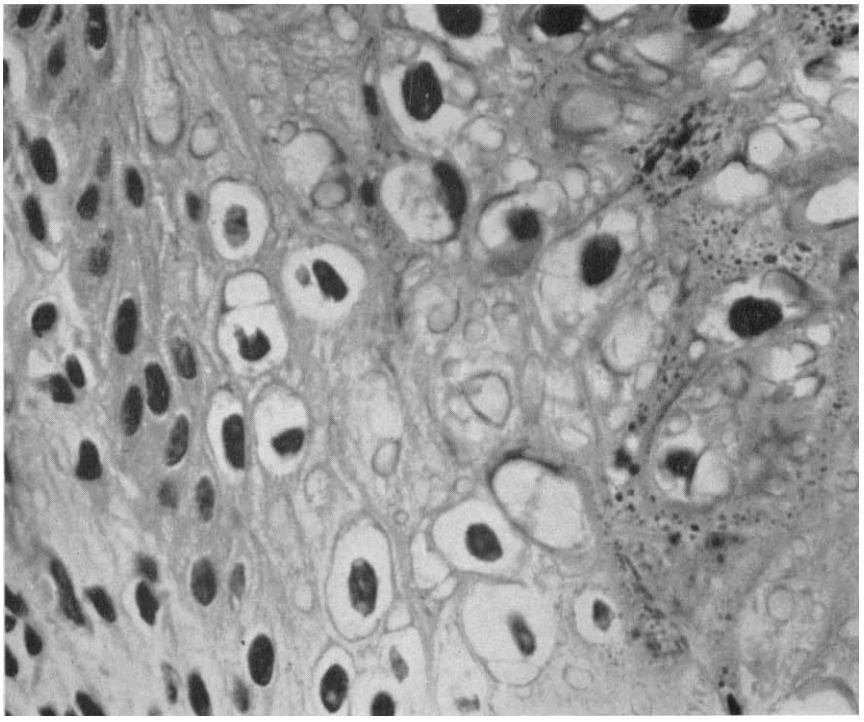

FIG. 3. Verruca vulgaris. H & E × 600. The epthelial cell nuclei contain varying amounts of basophilic material in the stages of formation of the "inclusion body." A few cells contain cytoplasmic keratohyalin granules.

Inclusions were found in warts removed from children and adults of all ages. Inclusion material seemed to occur more frequently in young, rapidly growing warts but were also found in ones which had been present for two years or more.

With the hematoxylin eosin and the pyronin-methyl green stained sections to serve as guides, a number of sections containing typical inclusion filled nuclei were examined after staining with toluidin blue (Figs. 7, 8). This dye stains both the desoxyribonucleic acids (DNA) and the ribonucleic acids (RNA) a blue color. All of the inclusion material within nuclei stained intensely blue indicating its high nucleoprotein content. Other possibly confusing granules such as keratohyalin failed to stain.

To demonstrate the presence of DNA alone in the inclusion material, Feulgen-

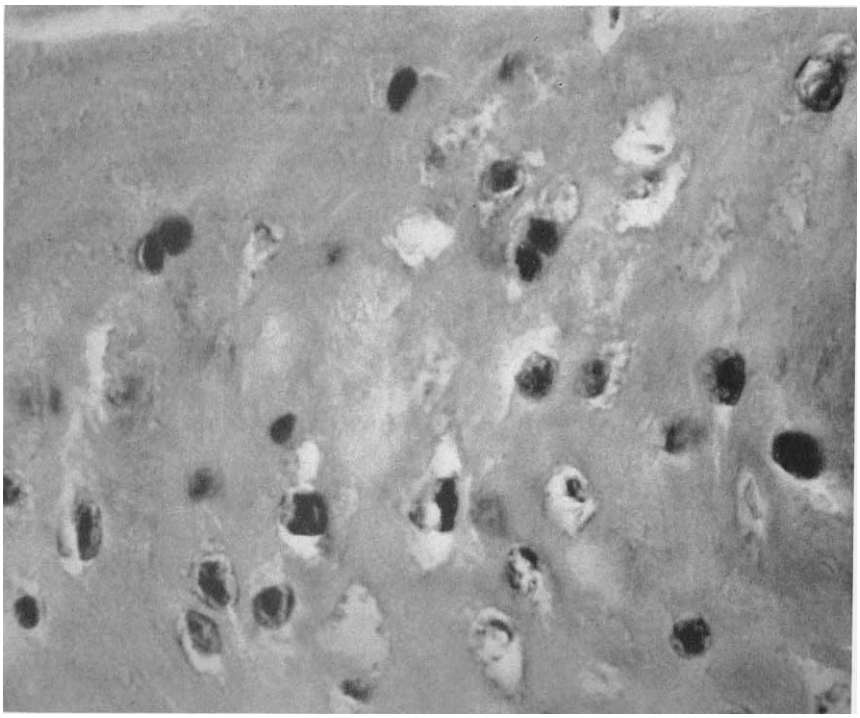

FIG. 4. Verruca vulgaris. H & E × 600. Additional examples of stages in the accumulation of nuclear inclusion material. Only the fully distended nucleus (mature "inclusion body") should be used for diagnostic purposes: (Cf. Fig. 6).

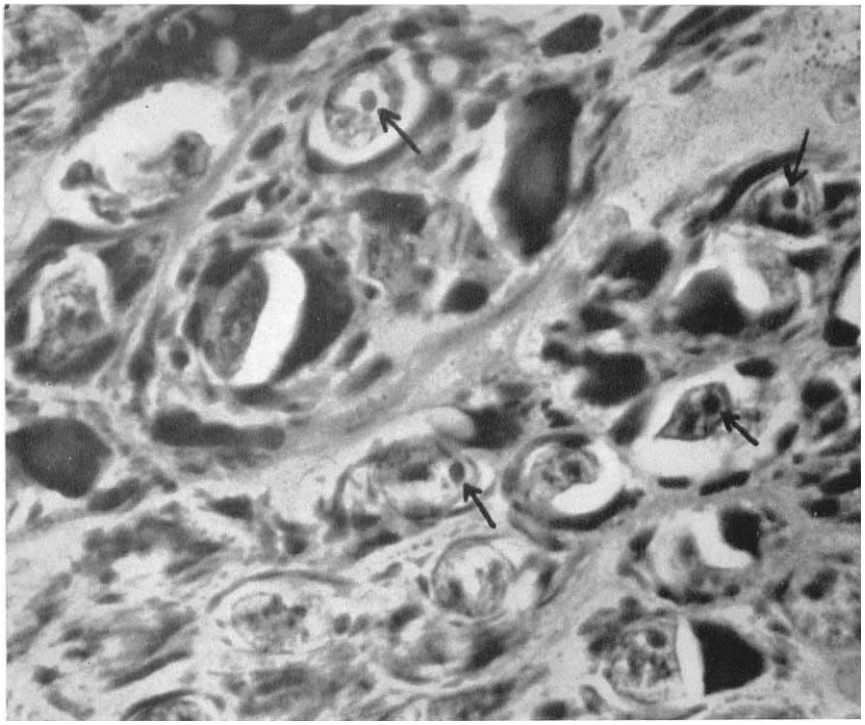

FIG. 5. Verruca vulgaris. H & E × 600. No diagnostic "inclusion bodies" are seen. Large nucleoli indicating nucleoprotein synthesis are indicated by arrows. Varying sized dense masses of keratohyalin frequently cause confusion.

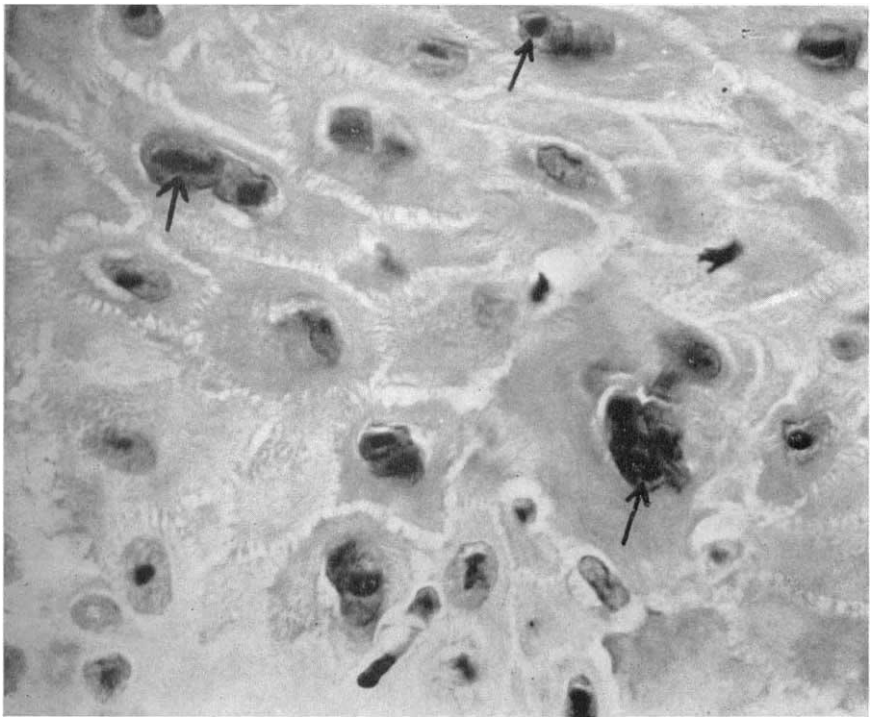

FIG. 6. Senile keratosis. H & E × 600. These nuclei contain abnormally large nucleoli (arrows). Of all sections of non-virus infected tissues examined, these most closely resembled verruca "inclusion bodies." The fully distended wart nuclei, however, are readily distinguished (Cf. Figs. 4, 8, 9).

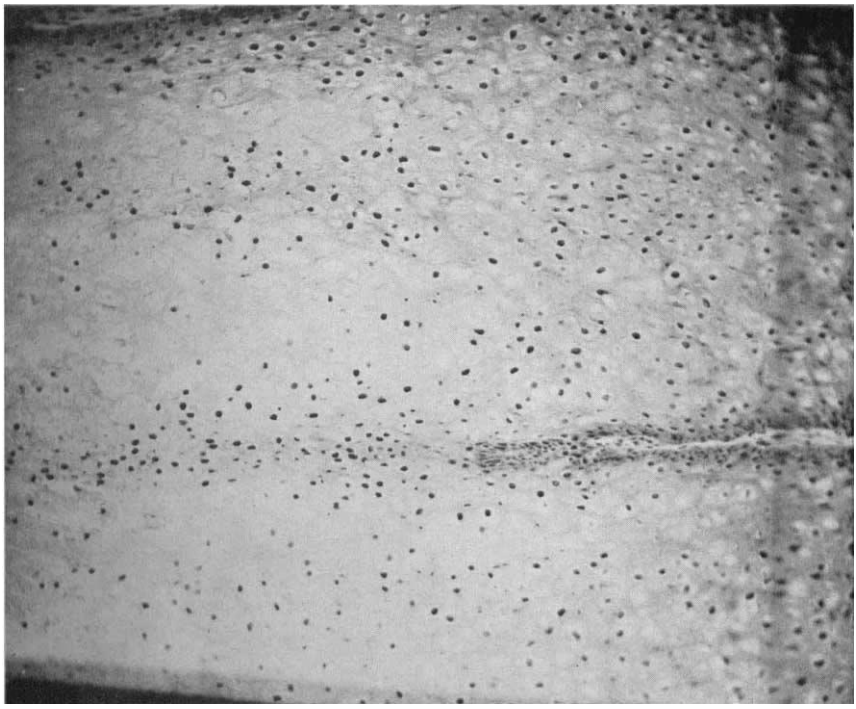

FIG. 7 Verruca vulgaris. Toluidin blue × 75. All nuclei stain blue but the "inclusion bodies" are larger and more dense than the deeper prickel cell and basal cell nuclei.

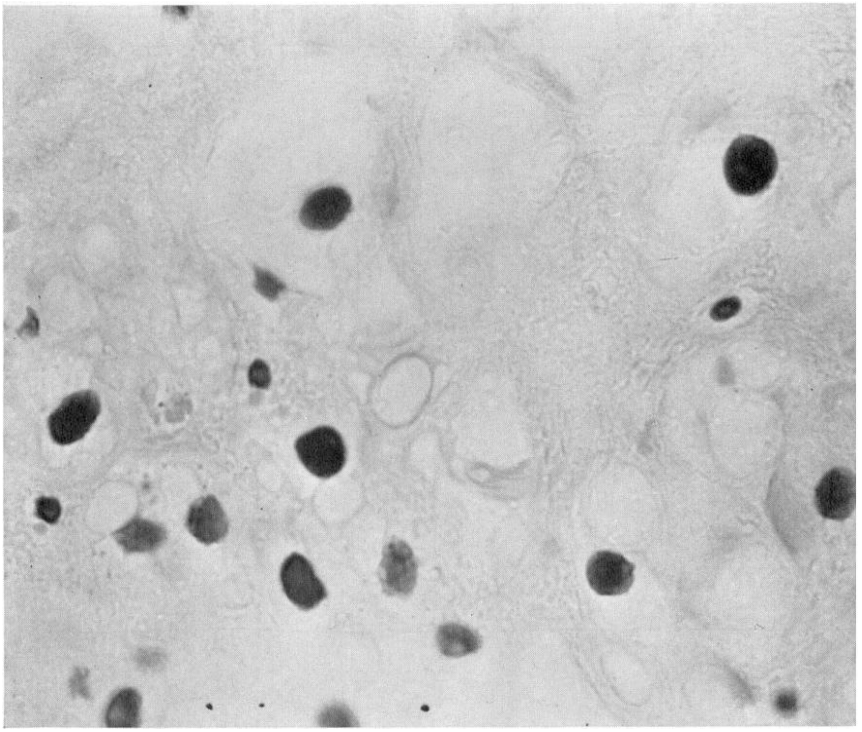

FIG. 8 Verruca vulgaris. Toluidin blue × 600. Varying amounts of dark staining nucleoprotein have accumulated in the nuclei. Some nuclei are almost normal while others are completely filled with inclusion material.

FIG. 9 Verruca vulgaris. Feulgen stain × 75. The intensity of staining of the "inclusion bodies" appears to be the same as with toluidin blue, which suggests that the nucleoprotein is all of the desoxyribose (DNA) type.

stained sections from the same verrucae were studied (Figs. 9, 10). These showed very heavy staining of the inclusions. Using structures known to contain only DNA nucleic acids such as chromatin and nuclear membrane as a guide, the

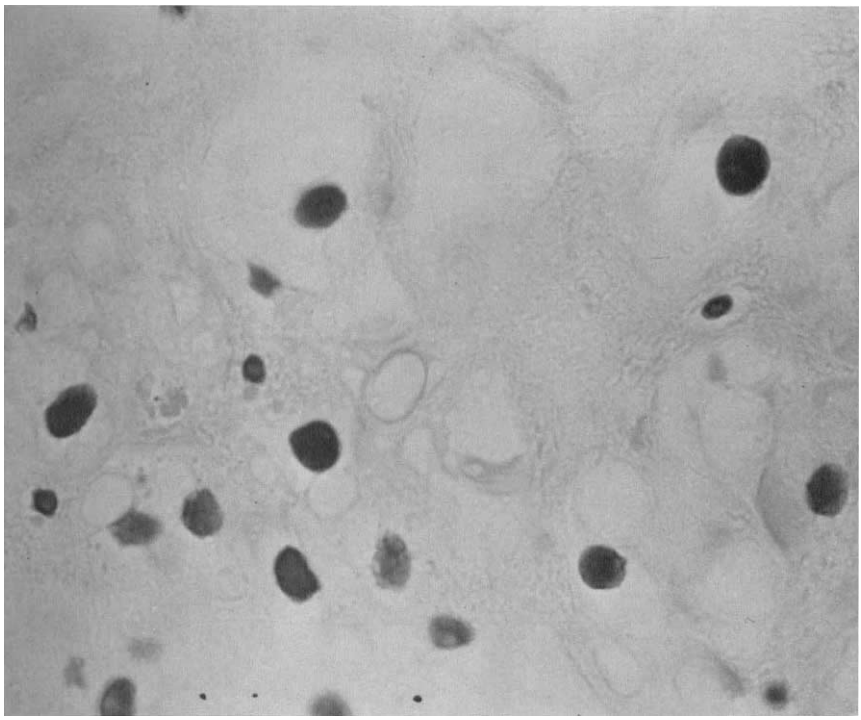

FIG. 10 Verruca vulgaris. Feulgen stain × 600. In some nuclei the nuclear membrane can still be distinguished from the DNA which distends and fills it, forming the "inclusion body."

TABLE I

CLINICAL TYPE OF VERRUCA	NUMBER OF PATIENTS FROM WHOM SPECIMENS EXAMINED	NUMBER WITH CHARACTERISTIC INCLUSION MATERIAL (H & E STAIN)
Vulgaris	51	28
Palmaris	1	1
Plantaris	1	1
Condyloma acuminatum	6	2
Plana	2	0
Filiformis	4	2
Totals	65	34

intensity of staining of the inclusion material indicated that the Feulgen stain was as intense as the toluidin blue stain. This suggests that most, if not all, of the inclusion material nucleic acids are the DNA form.

DISCUSSION

The clinical value of demonstrating characteristic inclusion bodies with hematoxylin and eosin in a histologic section is apparent. It should be possible, by demonstrating the distended inclusion filled nuclei, to make an unequivocal diagnosis of verrucae which is based on a more specific change than histologic arrangement. For diagnostic purposes, only the unmistakable fully developed inclusion should be reported, however (Fig. 6). At the present time we are unable to account for the absence of inclusions from some warts, although this may be due to their spotty distribution in a lesion and our failure to cut serial sections. Strauss, et al (14) have suggested that lesions containing inclusions are distinctive papillomas distinguishable from warts. We were unable to make this clinical differentiation.

Our studies confirm those of Lipschütz and Hyden. Although there are theoretical objections to postulating a dynamic process such as the development of an inclusion body on static cytologic evidence alone, the assumption that progression occurs as the epithelial cells mature and move to the surface seems reasonable. As Hyden has indicated, there is no question that one can find all stages of accumulation of intranuclear inclusion material, from small amounts which might be confused with chromatin, to the stage where it completely replaces all other nuclear structures.

The development of this aggregation of DNA in warts would seem to reflect the multiplication of virus by analogy with chemical studies which indicate that DNA is the chief nucleic acid constituent of the larger animal viruses (Hyden and Knight). The postulated stages of development of inclusion material in warts is lent further weight by the demonstration of this process in the cytoplasmic inclusions of vaccinia (Bland & Robinow) (1) and of molluscum contagiosum (Hyden (5), and Rake and Blank (11)), and by similar studies on the early intranuclear inclusions of herpes simplex (Crouse, Coriell, Blank and Scott (4)).

SUMMARY AND CONCLUSIONS

1. Infected epithelial cells of verrucae undergo cytologic changes distinct from all other diseases of the skin.

2. The pathognomonic change occurs in the nucleus. This consists of the accumulation of basophilic material (when stained with hematoxylin and eosin) which eventually fills and distends the nuclear membrane, and replaces all other nuclear structures.

3. The nucleus when completely filled with this inclusion material comprises the inclusion body of Lipschütz, and probably represents the culmination of the dynamic process of virus multiplication.

4. Approximately one-half of all verrucae examined contained cells with this inclusion material in their nuclei.

5. The inclusion material contains large amounts of nucleoprotein, most of which appears to be of the desoxyribose (DNA) form.

6. It is postulated that the accumulation of distinctive, newly-formed DNA

containing inclusion material is cytologic evidence of the aggregation of wart virus in the nucleus of epithelial cells.

7. As Hyden pointed out, the cytochemical changes in verrucae graphically demonstrate one of the ways in which a "virus parasitizes the nucleoprotein forming parts of the host cell."

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DISCUSSION

DR. MAURICE J. STRAUSS: I do not believe that Dr. Blank and his co-workers and our group are so far apart after all. It seems to me that we are not talking about the same thing. Dr. Blank has pointed out two types of verrucae, one in which they find nothing unusual and the second in which they find these accumulations of nuclear material which Lipschütz has described as nuclear inclusion bodies. We believe that there is a third type which I have described

at length a short while ago. The first low-magnification slide which Dr. Blank showed corresponds, I think, with the lesions we have studied. The rest of his slides I believe were something different (the second type that he has pointed out). One other difference seems apparent to me; that is the incidence. Dr. Blank reported the Lipschütz inclusion body-like findings in 28 out of 58 cases. We have found the changes which we have just reported in a much lower percentage.

I am certain that we have seen the lesions which Dr. Blank has just shown us but we have not done Feulgen stains on them. On my return to New Haven this will be done and I have no doubt that our findings will corroborate his. I hope that Dr. Blank will be able to identify the block of tissue which yielded the section from which his first slide was made because I believe that further study of that particular lesion should yield results similar to ours. I feel certain that we will eventually reconcile our apparent differences.

DR. STEPHEN ROTHMAN: I would like to ask about the use of the term "parakeratosis"? The degeneration forms shown here certainly are not in conformity with the current concept of parakeratosis.

DR. HARVEY BLANK: The unique nuclear changes in the epithelial cells of warts which we have demonstrated today have been observed for a long time by dermato-histopathologists. In the past they were most often called parakeratotic nuclei and were given little consideration. In the light of present studies, however, they are unique collections of inclusion material of a nucleic acid type indicative of the formation of new material, presumably newly forming virus. They are to be distinguished, therefore, from the degenerative nuclei seen in ordinary parakeratosis. We feel that some of the bodies within the nucleus which Dr. Strauss indicated in his original paper in the Proceedings of the Society of Experimental Biology and Medicine are the unusually active nucleoli indicative of new nucleic acid formation. Our findings are in agreement with his, however, in that the inclusion bodies which we describe are present in only a certain percentage of all warts. We have no explanation for our failure to find these bodies in all biopsies taken.